

Characterization of *Clostridium perfringens* Iota-Toxin Genes and Expression in *Escherichia coli*

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The iota toxin which is produced by *Clostridium perfringens* type E, is a binary toxin consisting of two independent polypeptides: Ia, which is an ADP-ribosyltransferase, and Ib, which is involved in the binding and internalization of the toxin into the cell. Two degenerate oligonucleotide probes deduced from partial amino acid sequence of each component of *C. spiroforme* toxin, which is closely related to the iota toxin, were used to clone three overlapping DNA fragments containing the iota-toxin genes from *C. perfringens* type E plasmid DNA. Two genes, in the same orientation, coding for Ia (387 amino acids) and Ib (875 amino acids) and separated by 243 noncoding nucleotides were identified. A predicted signal peptide was found for each component, and the secreted Ib displays two domains, the propeptide (172 amino acids) and the mature protein (664 amino acids). The Ia gene has been expressed in *Escherichia coli* and *C. perfringens*, under the control of its own promoter. The recombinant polypeptide obtained was recognized by Ia antibodies and ADP-ribosylated actin. The expression of the Ib gene was obtained in *E. coli* harboring a recombinant plasmid encompassing the putative promoter upstream of the Ia gene and the Ia and Ib genes. Two residues which have been found to be involved in the NAD⁺ binding site of diphtheria and pseudomonas toxins are conserved in the predicted Ia sequence (Glu-14 and Trp-19). The predicted amino acid Ib sequence shows 33.9% identity with and 54.4% similarity to the protective antigen of the anthrax toxin complex. In particular, the central region of Ib, which contains a predicted transmembrane segment (Leu-292 to Ser-308), presents 45% identity with the corresponding protective antigen sequence which is involved in the translocation of the toxin across the cell membrane.

Clostridium perfringens is a ubiquitous bacteria which causes food-borne illness and gas gangrene in humans and digestive diseases in animals. This species is divided into five toxin types on the basis of the production of four major lethal toxins (alpha, beta, epsilon, and iota) (6). *C. perfringens* type E produces the iota toxin and has been implicated in calf and lamb enterotoxemias (6).

Iota toxin is a binary toxin which is composed of two independent polypeptide chains called iota a (Ia) (M_r , 47,500) and iota b (Ib) (M_r , 71,500), which are not associated by either covalent or noncovalent bonds (26, 27). It has been shown that the Ia light chain causes ADP-ribosylation of globular skeletal muscle and nonmuscle actin at Arg-177 (23, 28). The heavy-chain Ib is required for penetration of Ia into the cytosol, and Ib must undergo limited proteolysis to be functionally active (3).

Iota toxin shares a comparable structure and mode of action with both *Clostridium spiroforme* toxin and C2 toxin of *Clostridium botulinum* types C and D (3). However, iota toxin is antigenically related to *C. spiroforme* toxin but not to *C. botulinum* C2 toxin (16, 19). An ADP-ribosyltransferase antigenically related to the enzymatic component of iota and *C. spiroforme* toxins was found in one *Clostridium difficile* strain (18). Iota and C2 toxins, like leucocidin (15), *Staphylococcus* gamma lysin (5), and anthrax (13) toxins, belong to the binary toxins formed of independent enzymatic and binding components. Therefore, this toxin family differs from the classical A-B toxins, such as the cholera and related toxins, which consist of a subunit or domain (A), with a specific function, and a binding domain or subunit(s) (B), and are assembled in a defined structure (25). In contrast, the protective antigen (PA) of the anthrax toxin binds to a cell

surface receptor, is cleaved by a cell surface protease, and then is able to bind the enzymatic component (edema factor or lethal factor) (13).

Little is known about the genetics of the *Clostridium* binary toxins. In the present study, we report the characterization of the iota-toxin genes and the relationship with anthrax toxin genes. We also studied their expression in recombinant *Escherichia coli* and *C. perfringens*.

MATERIALS AND METHODS

***C. spiroforme* toxin protein sequencing.** Light (Sa) and heavy chains (Sb) of *C. spiroforme* toxin were purified from *C. spiroforme* NCTC 11493 as previously described (19). Purified toxin chains were run on a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Paris, France). After Coomassie blue staining and destaining, the protein bands were cut out and digested by trypsin. The peptides were separated by high-performance liquid chromatography, and one internal peptide of each toxin chain was microsequenced with a gas-phase protein sequencer (Applied Biosystems).

Bacterial DNA and plasmids. *C. spiroforme* NCTC 11493 and Cs246, and *C. perfringens* type E strain NCIB 10748 were grown in broth containing Trypticase (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine-HCl (0.5 g/liter) (pH 7.2) under anaerobic conditions. *Clostridium* genomic and plasmid DNAs were extracted and purified as previously described (17).

Plasmid pUC19 (Appligene, Strasbourg, France) and pACYC184 (New England Biolabs, Ozyme, Paris, France) were used for cloning in *E. coli* TG1 (Δ lac-pro) *thi supE hsdD5 F'* (*traD36 proAB*⁺ Δ lacZM15), and pJIR418 (24) was used for expression in *C. perfringens* S13 (24).

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Probes and hybridization conditions. Oligonucleotides were synthesized by the phosphoramidite method with a Cyclone Milligen automated DNA synthesizer. Genescreen Plus filters (New England Nuclear Research Products, du Pont Nemours, Paris, France) were pretreated with 200 µg of heat-denatured salmon sperm DNA per ml in 1 M NaCl–10% dextran sulfate–0.5% SDS–50 mM Tris-HCl (pH 7.5) at 40°C and then treated with a 5'-³²P-labeled oligonucleotide (10⁶ cpm/ml) in the same mixture overnight at 40°C. Filters were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 40°C for 2 h and exposed overnight to Fujii RX films.

The recombinant DNAs used as probes were labeled by the random method with the Multiprime kit (Amersham, Paris, France). The hybridizations were carried out with the Rapid Hybridization Buffer (Amersham) at 65°C for 4 h. The filters were washed in 0.1× SSC–0.1% SDS at 65°C for 1 h.

PCR amplification. One hundred nanograms of DNA was amplified by the polymerase chain reaction (PCR) in a total volume of 100 µl of 10 mM Tris-HCl (pH 8.3)–50 mM KCl–4 mM MgCl₂–0.1% bovine serum albumin–100 µM deoxynucleoside triphosphate–10 mM β-mercaptoethanol–50 pmol of each primer–2.5 U of *Taq* polymerase (Amersham). Reaction mixtures were denatured at 95°C for 2 min and then subjected to 30 cycles of denaturation (20 s at 94°C), annealing (20 s at 5°C below the theoretical melting temperature of the primers), and extension (20 s at 72°C) in a Prem III thermal cycler (Flobio, Paris, France).

Other molecular biology techniques. Ligation and preparation of plasmid DNA from *E. coli* were conducted as described by Maniatis et al. (14). Bacteria were transformed by electroporation (17). *C. perfringens* electroporation was done according to the method of Scott and Rood (22). T4 polynucleotide kinase and calf intestinal phosphatase were obtained from Boehringer-Mannheim France, other enzymes were obtained from Pharmacia (Paris, France), and the Erase-a-base was obtained from Promega (Coger, Paris, France). DNA was sequenced by the dideoxy chain terminator procedure with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

Potential biohazards associated with the experiments described had been approved by the French National Control Committee.

Expression of recombinant proteins. *E. coli* TG1 transformed with recombinant plasmid pUC19 or pACYC184 was grown in LB medium (14)–50 µg of ampicillin per ml to an optical density at 600 nm of 1. One milliliter of the cell suspension was pelleted in a microcentrifuge, resuspended in 100 µl of 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5), and sonicated. One to 20 µl was mixed with 5 µl of Laemmli sample buffer, boiled for 3 min, and loaded onto a 0.1% SDS–12% polyacrylamide gel (12).

For immunoblotting analysis, proteins were transferred electrophoretically to nitrocellulose (Hybond C; Amersham). The nitrocellulose was first incubated for 1 h in phosphate-buffered saline containing 5% milk and then incubated overnight at room temperature with 1:400 dilution of immunopurified Ia or Sb (18, 19) rabbit antibodies. Bound antibodies were detected with peroxidase-labeled protein A and the chemiluminescence kit provided by Amersham.

Gel assay for ADP-ribosylation. In vitro ADP-ribosylation assay were performed with G actin isolated from *Xenopus laevis* oocytes as described previously (18). The mixture for the polyacrylamide gel electrophoresis (PAGE) assay (total volume, 20 µl) contained 7 µg of oocyte actin, 100 mM

Sb	256	Y	L	S	S	Y	L	E	S	N	T	A
P131	5'	-TAT	TTI	TCI	TCI	TAT	TTI	GAA	TCI	AAT	ACI	GCI
b		TAT	GTA	TCA	AGT	TAT	TTA	GAA	TCA	AAT	ACC	GCT
Sb		G	D	P	Y	270						
P131		GGI	GAT	CCI	TAT	-3'						
b		GGT	GAC	CCA	TAT							
Sa	127	D	K	P	M	Y	V	Y	Y	F	E	S
P222	5'	-GAT	AAA	CCI	ATG	TAT	GTI	TAT	TAT	TTT	GAA	TCI
b		GAT	AAA	CCT	ATA	AAT	GTT	TAT	TAT	TTT	GAG	TCT
Sa		P	E	139								
P222		CCI	GAA	-3'								
b		CCA	GAG									

FIG. 1. Amino acid sequences of internal peptides of *C. spiroforme* toxin components Sb and Sa and oligonucleotide probes P131 and P222 complementary to these sequences, respectively, according to the *Clostridium* codon usage (see the text). The nucleotide sequences determined by DNA sequencing of *C. perfringens* type E (NCIB 10748) are shown in lines b. The amino acids are numbered according to the iota-toxin sequence.

HEPES, 5 mM ATP, 2.5 mM ADP-ribose, and 5 × 10⁵ cpm of ³²P-NAD (specific activity, 30 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). Between 1 and 10 µl of a fraction to be tested for enzymatic activity was added. After incubation for 1 h at 37°C, sample buffer was added and the preparation was fractionated by SDS-PAGE and processed by autoradiography.

Purification of the recombinant Ia. *C. perfringens* S13 harboring pMRP76 was grown in TGY under anaerobic conditions to an optical density at 600 nm of 1. Culture supernatant (1 liter) was precipitated by ammonium sulfate (70% saturation). The precipitate was dissolved in 30 ml of 10 mM Tris (pH 7.5), dialyzed, and applied to a column (2 by 15 cm) containing DEAE-Sepharose CL6B (Pharmacia) equilibrated with 10 bed volumes of the same buffer. The gel was washed and eluted with 0.1 M NaCl in the same buffer. The eluate was purified on an immunoaffinity column with rabbit antibodies against *C. perfringens* toxin component Ia as previously described (19). The column was eluted with 4 M guanidine hydrochloride (Sigma) in 10 mM Tris (pH 7.5). The eluted fractions were dialyzed against the Tris buffer.

Nucleotide sequence accession number. The nucleotide sequences reported in this paper have been submitted to the EMBL Data Library with accession number X73562.

RESULTS

Cloning of the iota-toxin genes. Several different peptides of *C. spiroforme* toxin chains Sa and Sb were microsequenced. Two oligonucleotides (P131 and P222), deduced from internal peptide sequences of Sb and Sa, respectively, were synthesized according to the *Clostridium* codon usage (31) and with inosine at the most degenerated positions (Fig. 1). These probes hybridized with total and plasmid DNA of *C. spiroforme* NCTC 11493 and Cs246 and *C. perfringens* NCIB 10748 (data not shown), indicating that iota-toxin genes are localized on plasmid DNA of these *Clostridium* strains. Since DNA was easier to prepare from *C. perfringens* than from *C. spiroforme*, we chose to clone iota-toxin genes from *C. perfringens* NCIB 10748.

The 2-kb *Hind*III DNA fragment of *C. perfringens* NCIB 10748 plasmid DNA recognized by P131 was cloned into

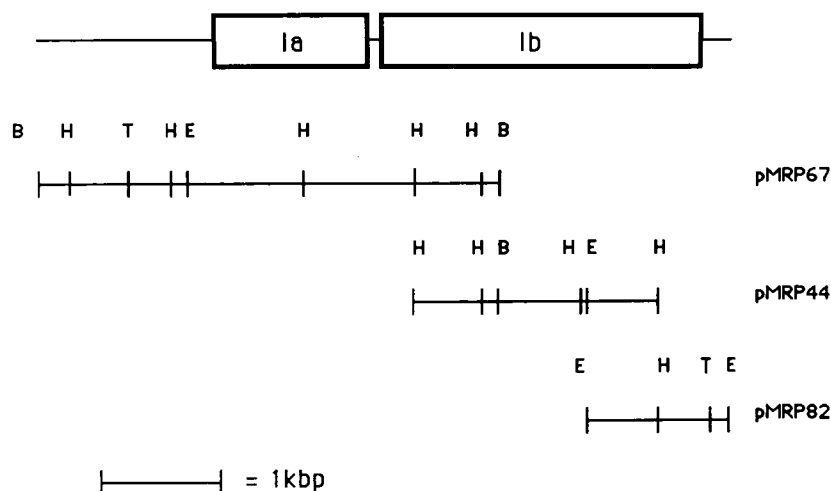


FIG. 2. Cloning strategy of the iota-toxin genes encoding the enzymatic Ia components and binding Ib components. B, *Bcl*I; E, *Eco*RI; H, *Hind*III; T, *Taq*I.

pUC19 (pMRP44). The synthetic oligonucleotide P190 complementary to the 5' extremity of the 2-kb *Hind*III DNA fragment (nucleotides 3288 to 3308) was used to clone the 3.8-kb *Bcl*I overlapping DNA fragment (pMRP67) (Fig. 2). A third clone (pMRP82), which contains the 1.2-kb *Eco*RI DNA fragment overlapping with the 3' extremity of the insert of pMR44, was obtained by using the primer P238 complementary to nucleotides 5141 to 5161.

The primer P222, deduced from the sequence of an internal peptide of Sa, hybridized with *C. spiroforme* and *C. perfringens* type E DNAs and also with pMRP67, suggesting that the Ia gene is in the proximity of the Ib gene.

The overlapping DNA fragments cloned in pMRP44, pMRP67, and pMRP82 were sequenced (Fig. 3). The DNA fragment cloned in pMRP44 was found to contain two internal *Hind*III sites, indicating a partial digestion of the DNA used for cloning or a rearrangement of the cloned *Hind*III DNA fragments. One of the internal *Hind*III sites was localized on the overlapping region with pMRP67 (Fig. 2 and 3). Two primers were synthesized on each part of the second *Hind*III site and in the opposite direction from P231 (positions 4638 to 4618) and P212 (positions 4225 to 4241) (Fig. 3). The PCR amplification products with these primers, *C. perfringens* DNA, and pMRP44 were the same size (data not shown). These results showed that the two internal *Hind*III sites in pMRP44 are the result of a partial DNA digestion.

Features of iota-toxin genes. The 5,747 nucleotide sequence showed two open reading frames. The open reading frame near the 5' extremity and recognized by P222, which was deduced from the Sa internal peptide sequence, was assigned to the Ia gene, and the downstream open reading frame, recognized by P131, was deduced from the internal Sb sequence and assigned to the Ib gene.

The Ia gene starts at the initiation codon ATG at position 1465 and ends at the stop codon at position 2628. A consensus ribosome binding site, GGAGGG, is localized 6 nucleotides upstream of the initiation codon. DNA stretches TATAAT and TTGTCAT, homologous to the -10 and -35 *Clostridium* consensus promoter regions (31), are identified between positions 1304 and 1333 (Fig. 3). Three direct repeats and one inverted repeat are localized in the promoter region (Fig. 3).

The Ib gene, from the initiation codon ATG (position 2872) to the stop codon at position 5499, is preceded by a consensus ribosome binding site (GGAGG) between positions 2860 and 2864. The Ib gene is located on the same frame as the Ia gene. On the 243 noncoding nucleotides between the two genes, no promoter consensus sequence has been found. No long inverted repeats have been identified downstream of the Ia and Ib genes.

Analysis of the deduced amino acid sequences. (i) **Ia sequence.** The deduced polypeptide is composed of 387 amino acids and has a predicted molecular mass of 45,121 Da. The 41 N-terminal amino acids contained residues characteristic of a signal peptide (20): the presence of charged N-terminal residues at positions 2, 3, and 6 followed by a long hydrophobic core, of a turn residue (Pro-23), and of a proteolytic cleavage site (Arg-41 to Ala-42). Furthermore, the amino acid sequence from Ala-42 to Glu-56 is in agreement with the sequence determined by protein sequencing of the N-terminal part of Sa (X-X-Ile-Glu-Pro-Pro-Pro-Asp-Phe-Leu-Lys-Lys-Lys-Glu). The discordant residues could be due to differences between Sa and Ia or to inaccuracies in protein sequencing (the presence of contaminating proteins or breakdown products which interfere with sequence analysis). However, the cleavage sites of the clostridial signal peptidases have not been fully investigated. It is not excluded that the cleavage by signal peptidase occurs at Ala-42, or Ala-34 with a subsequent cleavage at Arg-41 to Ala-42 by clostridial peptidase, since Ala-X represents a common site of bacterial signal peptidases (29).

The mature Ia protein is formed of 346 amino acids. The predicted molecular mass (40,392 Da) is comparable to that found for purified native Sa (44,000) and slightly lower than that of native Ia (47,500). The predicted pI of 5.01 agrees with the experimentally determined pI of Ia (5.2) and is lower than that of Sa (pI 6.2) (19, 26). The differences in the predicted and experimentally determined molecular masses of Ia could be due to a particular conformational structure.

The Ia sequence did not show any significant similarity with other known protein sequences by FASTA and Blast programs. Domenighini et al. have identified conserved amino acids involved in the NAD⁺ binding site of ADP-ribosylating toxins (4). Recently, it has been shown that the peptide Phe-9 to Gly-19 of the ADP-ribosylating enzyme C3

FIG. 3. Nucleotide sequence and amino acid translation of the iota-toxin genes. The putative Shine Dalgarno (S.D.) and -35 and -10 promoter sequences are underlined. The predicted signal peptides are italicized. Stop codons are indicated by an asterisk, and the directed and inverted repeats on the promoter region are indicated by dashed arrows beneath the sequence.

4075 ATG TAC AAA GTA ACT CCA ACA ACC AAT TTA GTA TTA GAT GGA GAG ACA
 418 L A T I K A Q D N Q I G N N L S
 4123 TTA GCA ACT ATT AAA GCA CAG GAT AAT CAA ATT GGT AAT AAC TTA TCT
 434 P N E T Y P K K G L S P L A L N
 4171 CCA AAT GAA ACA TAT CCT AAA AAA GGA CTT TCT CCT TTA GCT CTT AAC
 450 T M D Q F N A R L I P I N Y D Q
 4219 ACA ATG GAT CAA TTT AAT GCT AGG TTA ATT CCA ATA AAT TAC GAT CAA
 466 L K K L D S G K Q I K L E T T Q
 4267 CTT AAA AAA TTA GAT TCT GGA AAA CAA ATT AAA TTA GAA ACA ACA CAA
 482 V S G N Y G T K N S Q G Q I I T
 4315 GTA AGT GGA AAT TAT GGA ACT AAA AAT AGT CAA GGA CAA ATA ATT ACA
 498 E G N S W G S N Y I S Q I D S V S
 4363 GAA GGA AAT AGT TGG TCT AAT TTA ATA AGT CAA ATT GAT AGC GTT TCT
 514 A S I I L D T G S Q T F E R R V
 4411 GCA TCT ATT ATA CTG GAT ACA GGA AGT CAA ACA TTT GAA AGG CGT GTG
 530 A A K E Q G N P E D K T P E I T
 4459 GCT GCT AAA GAG CAA GGA AAT CCA GAA GAT AAA ACT CCT GAG ATT ACA
 546 I G E A I K K A F S A T K N G E
 4507 ATT GGA GAA GCA ATT AAA AAA GCT TTT AGT GCT ACT AAA AAT GGT GAA
 562 L L Y F N G I P I D E S C V E L
 4555 TTA TTA TAT TTT AAT GGA ATT CCA ATT GAT GAG AGC TGT GTT GAA CTT
 578 I F D D T S E I I K E Q L K Y
 4603 ATA TTT GAT GAT AAT ACA TCT GAA ATA ATT AAA GAA CAA TTA AAA TAT
 594 L D D K K I Y N V K L E R G M N
 4651 TTA GAT GAT AAA AAG ATA TAT AAT GTT AAA CTT GAA AGA GGA ATG AAT
 610 I L I K V P S Y F T T N F D E Y N
 4699 ATA TAT AAA AAG GTG CTT TCA TAT TTT ACT AAT TTT TAT GAT AAT AAT
 626 N F P A S W S N I D T K N Q D G
 4747 AAT TTT CCT GCT TCA TGG AGT AAT ATT GAT ACT AAA AAC CAA GAT GGT
 642 L Q S V A N K L S G E T K I I I
 4795 TTA CAA AGT GTA GCA AAT AAG TTA AGC GGA GAG ACA AAG ATT ACA ATA
 658 P M S K L K P Y K R Y V F S G Y
 4843 CCT ATG TCT AAA TTA AAA CCC TAT AAA CGC TAT GTT TTT AGT GGA TAT
 674 S K D P S T S N S I T V N I K S
 4891 TCA AAG GAT CCT TCA AAT TCT AAT TCA ATA CAA GAT AAT AAA TCA
 690 K E Q K T D Y L V P E K D Y T K
 4939 AAA GAA CAG AAA ACA GAT TAT TTA GTA CCA GAG AAA GAT TAT ACA AAA
 706 F S Y E F E T T G K D S S D I E
 4987 TTT AGT TAT GAA TTT GAA ACA ACC GGA AAA GAT TCT TCT GAT ATA GAA
 722 I T L T S S G V I F L D N L S I
 5035 ATA ACA TTA ACA AGT AGT GGT GTA ATA TTT TTA GAT AAT TTA TCT ATT
 738 T E L N S T P E I L K E P E I K
 5083 ACA GAA TTA AAT AGT ACT CCT GAA ATA TTA AAA GAA CCA GAA ATT AAA
 754 V P S D Q E I L D A H N K Y Y A
 5131 GTT CCA AGT GAC CAA GAA ATA CTA GAT GCA CAT AAC AAA TAT TAT GCA
 770 D I K L D T N T G N T Y I D G I
 5179 GAT ATA AAG CTT GAC ACA AAT ACA GGA AAC ACT TAT ATA GAT GGT ATA
 786 Y F E P T Q T N K E A L D Y I Q
 5227 TAT TTT GAA CCA ACT CAA ACT AAT AAA GAA GCT CTT GAT TAT ATT CAA
 802 K Y R V E A T L Q Y S G F K D I
 5275 AAA TAT AGA GTT GAA GCA ACT TTG CAA TAT TCA GGA TTT AAA GAT ATT
 818 G T K D K E I R N Y L G D Q N Q
 5323 GGA ACT AAG GAT AAA GAA ATA CGT AAT TAT TTA GGA GAT CAA AAC CAA
 834 P K T N Y I N F R S Y F T S G E
 5371 CCT AAA ACT AAT TAT ATT AAT TTT AGA AGT TAT TTT ACT AGT GGA GAA
 850 N V M T Y K K L R I Y A V T P D
 5419 AAT GTT ATG ACA TAT AAA AAA TTA AGA ATA TAT GCA GTT ACA CCT GAT
 866 N R E L L V S V N *
 5467 AAT AGA GAG TTA TTA GTG CTT AGT TAT TAA TACTAAATAAAATATATTT
 5520 TAAATTTATTAATAATCAAAATTAATAAGCTAGTTATTCATCTGGCTACCTATATATAAT
 5584 ATTTCTCGATTACTTACAGAGGATGCAATAATTTTGTTGAATATCTAAAGAAATATATTGA
 5648 AATTTGTTGAGTTTATGATAATATTTTATTTAAACCTTAATTTCTTTTATGAATATATAGTG
 5712 AATATAAATTTGACAAAACCTAGATATTTGAATTC

FIG. 3—Continued.

from *C. botulinum* C-003-9 constitutes the adenine ring-binding domain of the NAD⁺ binding site (1). In this peptide, Glu-13 and Trp-18 align with Glu-148 and Trp-153 of diphtheria toxin (DT), Glu-553 and Trp-558 of pseudomonas

Iota	10-L K D K E N A I Q W E K-21
DT	144-S S S V E Y I N N W E Q-155
ET	549-G G R L E T I L G W G N-560
PT	17-D V F Q N G F T A W G N-28
C3 (C-003-9)	9-F T N V E E A K K W G N-20
C3 (C-468)	9-F T N I D Q A K A W G N-20
EDIN	9-F T D L D E A T K W G N-20
consensus	E/D X X X X W

FIG. 4. Alignment of the Ia amino acid sequence with those involved in the NAD⁺ binding site of DT, ET, PT, C3 enzyme from *C. botulinum* C-003-9, from *C. botulinum* C-468, and from *Staphylococcus* EDIN. The conserved amino acids which have been assigned to cross-link the nicotinamide ring of NAD⁺ in DT, ET, PT, and the corresponding residues in the other sequences are shown in boldface.

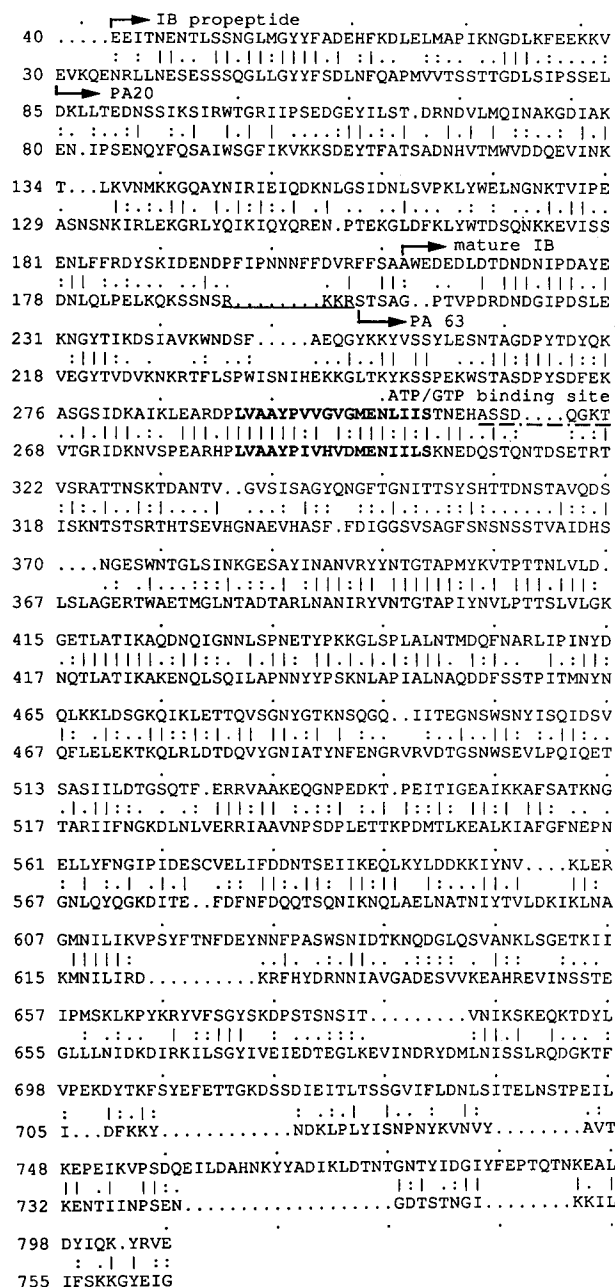
exotoxin A (ET), and Trp-26 of pertussis toxin (PT), which have been assigned to cross-link the nicotinamide ring of NAD⁺ (4). In the C3 enzyme of *C. botulinum* C-468 (17) and the epidermal cell differentiation inhibitor (EDIN) of *Staphylococcus aureus* (8), which is a related enzyme, Glu-13 is replaced by the equivalent residue Asp-13 (Fig. 4). By comparing the Ia sequence with those of the other ADP-ribosylating toxins, Glu-14 and Trp-19 of Ia can be aligned with the above conserved residues (Fig. 4). As for C3, EDIN, and PT, these conserved residues in Ia are localized near the N terminus.

(ii) **Ib sequence.** The Ib 875 deduced amino acids have a predicted molecular mass of 98,467 Da. The N-terminal 39 amino acids form an hydrophobic domain with an N-terminal-charged residue (Lys-6) and probably correspond to a peptide signal. A potential cleavage site for signal peptidase (Lys-39 to Glu-40) is localized at the extremity of this putative signal peptide. The predicted precursor protein consists of 836 residues (94,013 Da) and is in agreement with that found for the Sb precursor (92,000 Da) (19). The N-terminal sequence of the naturally activated form of Sb determined by protein sequencing (Gly-Trp-Gly-Asp-Glu-Asp-Leu-Asp) matches amino acids Ala-212 to Asp-219. The two unrelated residues could correspond to differences in Ib and Sb sequences or inaccuracies in protein sequencing. The deduced polypeptide from Ala-212 to Asn-875 (664 amino acids) is predicted to correspond to the functional proteolytically activated form of Ib or mature Ib. The predicted molecular mass (80,890 Da) and pI (4.67) are in agreement with those determined experimentally for Sb (76,000 Da and pI 4.7) (19) and differ slightly from those found for Ib (67,000 Da and pI 4.2) (26).

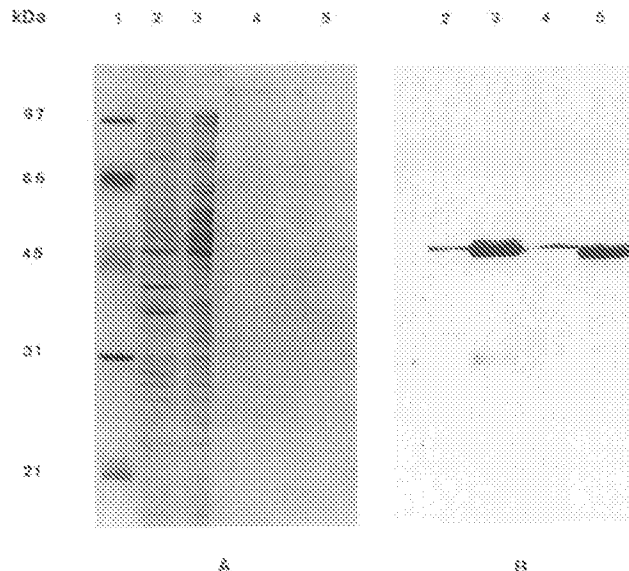
The propeptide (Glu-40 to Ala-211), which is excised from the Ib precursor, consists of 172 amino acids, with a predicted molecular mass of 19,883 Da and a pI of 4.62.

Similarity between Ib and PA. By comparison with protein sequences available in the data bank, Ib displays significant homology with only the PA of the anthrax toxin complex (13). Overall 33.9% identity and 54.4% similarity have been found between both protein sequences. The central regions of these proteins show the maximum homology, 45% identity and 70% similarity between amino acids 268 to 531 of Ib and amino acids 260 to 536 of PA.

Ib and PA present similar structures. Both contain a signal peptide (39 residues for Ib and 29 residues for PA), a propeptide (19,883 Da for Ib and 19,243 Da for PA), and a mature protein (80,000 Da for Ib and 63,000 Da for PA) (13).



It has been shown that the cleavage between the propeptide and mature PA is carried out by a cellular membrane protease with the specificity of furin at the site 164-Arg-Lys-Lys-Arg-167 (Fig. 5) (11). A consensus furin cleavage site has not been found in the Ib sequence.



The hydrophobic sequence Leu-292 to Ser-309 of Ib is predicted to form a transmembrane segment by using the program described by Klein et al. (9). This segment shares a high degree of homology with a sequence found in PA: Leu-284 to Leu-300 (12 identical residues of 17) (Fig. 5). The hydrophobic PA segment is predicted to form an uncertain transmembrane segment (prediction by the quadratic function but not by the linear function of the program of Klein et al. [9]). These data suggest that these sequences could constitute a common functional domain in Ib and PA which could probably be involved in the translocation of the toxin across the cell membrane.

An ATP/GTP binding [consensus GXXXXGK(TS)] (21) has been identified in the Ib sequence from positions Ala-314 to Thr-321 (ASSDQGKT) (Fig. 5). This motif has not been found in the PA sequence.

Expression of Ia gene in recombinant *E. coli* and *C. perfringens*. *E. coli* TG1 harboring the recombinant plasmid pMRP67 and *C. perfringens* S13 harboring pMRP76 (corresponding to the shuttle vector pJIR418 with the pMRP67 insert cut by *SacI*-*SalI* localized on the multiple cloning sequence) were analyzed for expression of the Ia gene by Western blotting (immunoblotting) and ADP-ribosylation of actin. As shown in Fig. 6, a 44-kDa peptide recognized by antibodies raised against native purified Ia was found in cell extracts of the recombinant *E. coli* and *C. perfringens* strains and in culture supernatant of *C. perfringens* harboring pMRP76. The affinity-purified Ia from the culture supernatant of the recombinant *C. perfringens* exhibited the same electrophoretic mobility as the native Sa (data not shown).

The pMRP91 corresponding to the subcloning into pACYC184 of the *EcoRI-BclI* fragment of pMRP67 also induced the production of the Ia component (Fig. 6). These results indicate that the promoter of the Ia gene is localized in the 245 nucleotides upstream of its start codon and that

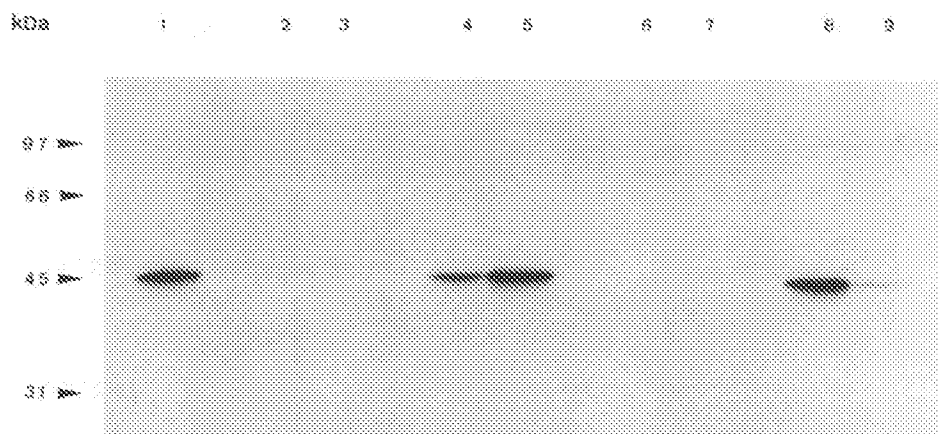


FIG. 7. Autoradiogram of analysis of Ia expression by ADP-ribosylation of actin as described in Materials and Methods. Native Sa as the positive control (lane 1) and cellular extract and culture supernatant of recombinant *C. perfringens* (pMRP76) (lanes 4 and 5, respectively), of recombinant *E. coli* (pMRP67) (lanes 8 and 9, respectively), of *C. perfringens* control (pJIR418) (lanes 2 and 3, respectively), and of *E. coli* control (pUC19) (lanes 6 and 7, respectively) are shown. Molecular mass markers are indicated on the left.

the 1,219 nucleotides upstream of the *Eco*RI site, in pMRP67, are not required for Ia gene expression. Although pMRP67 and pMRP76 contain a part of the Ib gene (DNA fragment encoding the signal peptide, propeptide, and the 99 N-terminal amino acids of the mature Ib), no Ib products, recognized by polyclonal antibodies against Ib, were observed by Western blotting (data not shown).

As shown in Fig. 7, cell extracts and culture supernatants of recombinant *E. coli* and *C. perfringens* strains exhibited ADP-ribosyltransferase activity with actin as the substrate. The enzymatic activity is greater in the cell extract of *E. coli* and the culture supernatant of *C. perfringens* than in the culture supernatant of *E. coli* and the cell extract of *C. perfringens*, indicating that the recombinant Ia is secreted by only *C. perfringens*. The time course of Ia production showed that Ia was released in *C. perfringens* culture supernatant early during the growth phase. In *E. coli*, however, low amounts were released in late growth phase, probably because of spontaneous cell lysis.

Total Ia production (in cell lysate and culture supernatant) was compared in *E. coli* and *C. perfringens* by using a similar concentration of bacteria (10^8 bacteria per ml, counted in a Malassez chamber) in stationary growth phase. Taking into account that the copy number of pJIR418 in *C. perfringens* is 15-fold less than that of pUC19 in *E. coli* (24), it can be estimated that *C. perfringens* produces 8-fold more Ia than *E. coli*.

Expression of the Ib gene in recombinant *E. coli*. The recombinant plasmid pMRP108 was constructed as shown in Fig. 8. This plasmid encompasses the putative promoter upstream from the Ia gene and the Ia and Ib genes. As shown in Fig. 9, the cell extract of *E. coli* TG1 harboring pMRP108 exhibited 90- and 60-kDa peptides recognized by specific anti-Sb antibodies. In length, the 90-kDa peptide corresponded to the recombinant mature Ib and its propeptide. But, the mature Ib is not processed in *E. coli* TG1, indicating that the cleavage site between the propeptide and mature Ib is not recognized by *E. coli* proteases. The Ia gene was also expressed in recombinant *E. coli* TG1 harboring pMRP108 as well as pMRP91 (data not shown).

DISCUSSION

We show that the two independent components Ia and Ib of the iota toxin are encoded by two genes separated by 243 noncoding nucleotides and preceded by a typical Shine-Dalgarno sequence. DNA sequence analysis suggests that the iota-toxin genes are under the control of the same promoter upstream of the Ia gene. A characteristic hydrophobic signal peptide has been identified at the N termini of both Ia and Ib sequences. This is in agreement with the fact that native iota toxin is exported from *C. perfringens* (27).

The iota-toxin sequence seems to be closely related to that of *C. spiroforme* toxin. The protein sequencing of the *C. spiroforme* toxin peptides, which correspond to a total number of 48 residues, shows 83.4% identity with the deduced amino acid sequence of the iota toxin. It has not been excluded that the identity level is higher, since protein sequencing is not as accurate as DNA sequencing. These data are in agreement with the close immunological relationship found between both toxins (16, 19).

The Ia gene has been expressed in *E. coli* and *C. perfringens* under the control of its own promoter. The Ia expression yield was higher in *C. perfringens* than in *E. coli*. The recombinant Ia was recognized by antibodies raised against native Ia and exhibited ADP-ribosyltransferase activity with actin as the substrate. Moreover, the recombinant Ia, in association with native Sb, induced morphological alterations of Vero cells as native iota toxin and *C. spiroforme* toxin did (data not shown). These data indicate that the Ia gene that we have identified corresponds effectively to the Ia structural gene.

The Ib gene was also expressed in *E. coli*, but the processing of the mature Ib did not occur in *E. coli*. It seems that the Ia and Ib genes form an operon under the control of a same promoter upstream from the Ia gene, since no promoter consensus sequence has been found upstream the Ib gene. The apparent lack of expression of the truncated Ib gene in pMRP67 and pMRP76 could be explained by an absence of epitopes recognized by the anti-Sb antibodies. Further work is needed to precisely determine the transcription of these two genes.

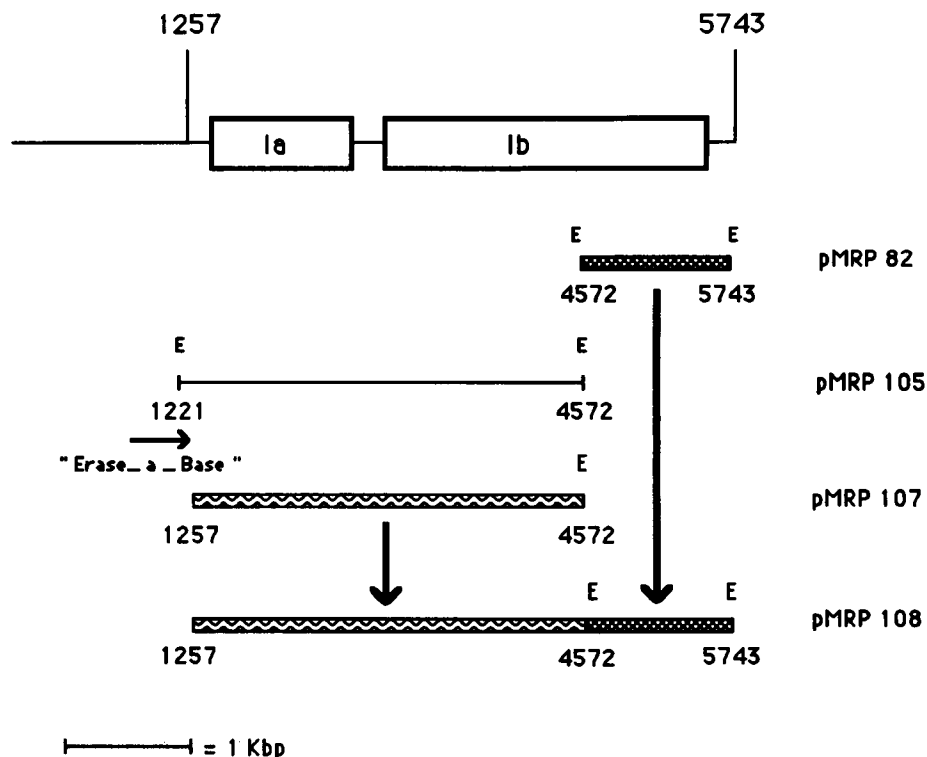


FIG. 8. Construction of recombinant plasmid for Ib expression. The 3,351-bp *EcoRI* fragment encompassing the Ia gene and most of the Ib gene was cloned from *C. perfringens* NCIB 10748 DNA into pUC19 (pMRP105). The *EcoRI* site upstream from the Ia gene was deleted by using the Erase-a-base kit, yielding pMRP107. The *EcoRI* insert of pMRP82 was cloned into the *EcoRI* site of pMRP107 (pMRP108). The numbers indicate the nucleotide positions from Fig. 3.

The Ia sequence shows two residues (Glu-14 and Trp-19) which can be aligned with amino acids involved in the NAD⁺ binding site of DT, ET, PT, and C3. The consensus motif (Glu/Asp)XXXX-Trp seems to be involved in the NAD⁺ binding site of these ADP-ribosylating toxins. How-

ever, Ia and C3 enzymes do not show a site equivalent to His-21 of DT, which is conserved in the other ADP-ribosylating toxins and has been found to be essential for ADP-ribosylation (4). As the substrate of ADP-ribosylation of Ia is an ATP-binding protein (actin) and a GTP-

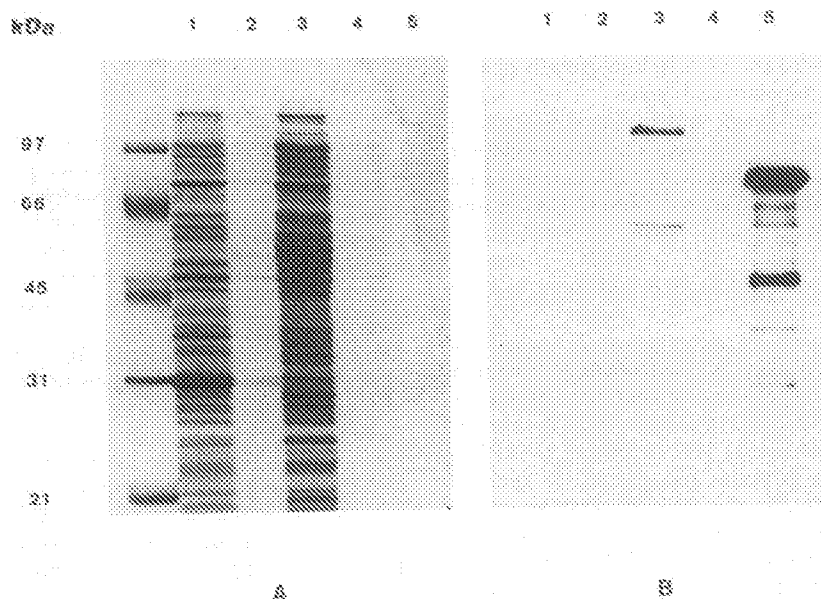


FIG. 9. Analysis of Ib expression in *E. coli*. (A) SDS-PAGE (12% polyacrylamide) of *E. coli* TG1 harboring pUC19 (lanes 1 and 2) or pMRP108 (lanes 3 and 4) (cell extract and culture supernatant, respectively) and of purified Sb (lane 5). (B) Western blot with specific anti-Sb antibodies. The lanes are the same as for panel A.

binding protein for the other ADP-ribosylating toxins, the NAD⁺ binding site could be slightly different in Ia.

The Ib protein can be divided in three domains (signal peptide, propeptide, and mature protein) defined by two protease cleavage sites. The presence of a propeptide located between the signal peptide and the mature protein is a common feature of most bacterial extracellular proteases, in which it is involved in the correct folding of the mature protein (30). But, no significant homology of Ib with known bacterial protease sequences has been found. The epsilon toxin produced by *C. perfringens* types B and D is secreted as a protoxin (32 kDa), which is trypsin activated by cleavage of 13 basic N-terminal amino acids (7). The propeptide of Ib (172 amino acids) is much longer, and its release does not induce a significant pI change in the protein.

Ib displays a similar structure and significant homology with the PA component of the anthrax toxin (13). Both proteins act as a binding component between cell surface receptors and the enzymatic component and trigger the toxin internalization allowing the delivery of the enzymatic component to its intracellular target. However, PA exhibits some modes of action different from those of Ib. The PA precursor binds to cell surface receptors (the cell recognition domain is located between residues 315 and 735) and is cleaved by a cell surface protease (furin). The propeptide is released, and the mature PA remains bound to the cell surface receptor and is able to bind either the enzymatic component edema factor (an adenylate cyclase) or lethal factor (a metalloprotease) (10, 13). Residues 168 to 312 of PA encompass the binding site for the edema factor and lethal factor (11, 13). In contrast, Ib needs to be proteolytically activated prior to being functional on cultured cells. These data suggest that the cell surface proteases are probably inactive on Ib. Moreover, the furin cleavage site (Arg-XX-Arg) has not been found in the Ib sequence. The cleavage site Ala-211 to Ala-212 between the propeptide and mature Ib, which has been determined by protein sequencing of the N terminus of mature Ib, corresponds to a common signal peptidase site (29).

The predicted transmembrane segment (Leu-292 to Ser-308) of Ib is highly homologous to the PA segment (Leu-284 to Leu-300) and could be involved in the toxin translocation across the cell membrane. Other binary toxins, such as DT, present hydrophobic helices predicted to be transmembrane segments in their translocation domain (2). In addition, the Ib sequence displays an ATP or GTP binding site (Fig. 5), which could be nonfunctional. However, it is not known whether ATP is required for the entry into the cells of iota toxin.

The relatedness between Ib and PA suggests that they could be derived from a common ancestor gene. Moreover, the GC contents of Ib and PA genes (28 and 31 mol%, respectively) are nearly the same. The central domain implicated in the translocation of the toxin across the cell membrane corresponds to the most conserved region, suggesting that both iota and anthrax toxins probably use a common mechanism of cell entry. The molecular analysis of the other binary toxin genes will provide further information about the evolution of this toxin family.

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